STUDY ON THE HYDROLYSIS PROCESS OF FOOD AND FEED SAMPLES USING HIGH PRESSURE ASHER (HPA-S) TO DETERMINE SEVERAL AMINO ACIDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract

The study researchs on the hydrolysis process of food and feed samples using High Pressure Asher (HPA-S) to determine several amino acids by high-performance liquid chromatography (HPLC). HPA-S equipment is mainly used for samples treatment in analysis of metals by spectroscopy. However, the study also found that HPA-S can be used in hydrolysis of food and feed samples in order to analyze amino acids. The temperature of HPA-S equipment could reach 300^{o}C and maintain continuously at 130 bar pressure, completely digetsing the most complex samples matrix within an hour. The successful study of the application of HPA-S to hydrolyze samples in order to analyze amino acids makes the time of sample preparation significantly shorter but still gave the equivalent stability, even higher than the common samples hydrolyzation.

Keywords: *food, feeding, amino acids, HPA-S, hydrolysis, HPLC.*

1. INTRODUCTION

The determination of amino acids mainly used derivatization method with some specific reagents and then could be analyzed by high-performance liquid chromatography with fluorescence detector. In most cases, the steps of sample preparation are complicated, taking so much time to make the hydrolysis of samples in a closed system using an oven or reflux heater (from 18 to 24 hours). Therefore, it is necessary to develop a simpler sample treatment method, shortening the sample hydrolysis time and achieving the same results.

In this study, the system of High pressure asher (HPA-S) specifically for samples digestion to analyze metal elements was used for the purpose of hydrolyzing amino acids in food and feeding samples. Then, amico acids were determined by high-performance liquid chromatography (HPLC). The research objective is to shorten the time of sample preparation but it still ensures the stability and effectiveness of the hydrolysis process.

2. SUBJECTS AND RESEARCH METHODS

2.1. Subjects

Subjects of this study were amino acids in food and feed samples after being hydrolyzed by means of HPA-S.

2.2. Equipment, tools and chemicals

The high pressure asher (HPA-S) system was from Anton paar, with the ability to maintain the maximum temperature of 300^{o}C and the maximum pressure of 130 bar. High-performance liquid

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chromatography system with fluorescent detector, and specific column for simultaneous analysis of 17 amino acids, C18 column normally for analysis of some essential amino acids was used in this study.

Pure chemicals meet the requirements for analysis, including was: HCl 37%, NaOH, and $Na_2B_4O_7.10H_2O$ from Merck. Standard solutions of amino acids and single standards of amino acids: Lysine (Lys), Arginine (Arg), Threonine (Thr), Glutamic acid (Glu) were from Sigma. The ACCQ amino acid derivative kit was from Waters. Other chemicals used for analysis such as Ammonium acetate, Methanol from Merck, O-phthaldialhehyd (OPA) and 2-mercaptoethanol (2-MCE) from Sigma. Distilled water was used. Nitrogen has reached the level of 99.999% purity.

In addition, other laboratory instruments such as centrifuge tubes, volumetric flasks, filter paper, indicator paper and other common tools were used.

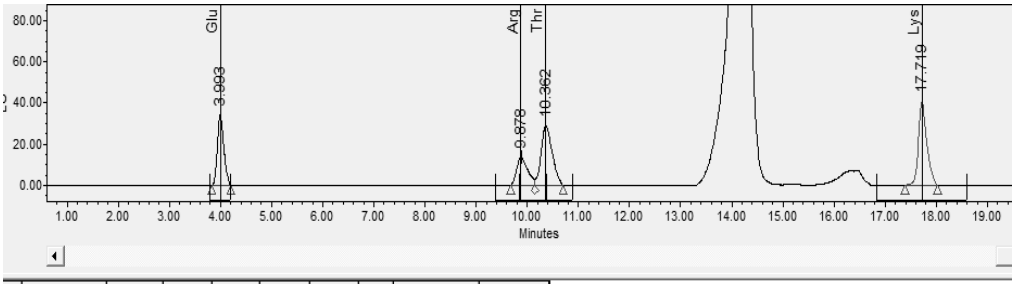
3. RESULTS AND DISCUSSION

3.1. Survey conditions for analyzing four essential amino acids (Lys, Arg, Thr, Glu)

To facilitate the evaluation of sample hydrolysis, due to the structure and similar chemical properties of amino acids, four essential amino acids including lysine, arginine, threonine, and glutamic acid were selected. Conditions for the separation and analysis of four amino acids simultaneously with C18 column using pre-column derivative with OPA reagents were surveyed with parameters that greatly affect the resolution, intensity and stability of analyte signal such as gradient mobile phase program and derivation time. The specific chromatographic conditions are described:

- C18 column (150 mm x 4.6 mm x 3.5 µm);
- Mobile phase: A: CH_3COONH_4 20 mM; B: Methanol, according to an appropriate gradient program to completely separate four amino acids.
- Flow rate: 0.5 mL/min
- Flourescence detector: λ_{ex}: 340 nm ; λ_{em}: 455 nm
- Injection: 20 µL
- Column temperature: 400^{0}C
- Derivation time: 60s

The chromatogram of four amino acid mixtures is shown in Figure 1.



Retention Time (min)	Area (µV*sec)	% Area	Height (µV)	Int Type	Amount	Units	Peak Type	Peak Codes
3.993	2999828	21.23	340289	BB			Unknown	
9.878	2077772	14.71	138793	BV			Unknown	
10.362	4395563	31.11	289309	VB			Unknown	
17.719	4654064	32.94	405342	BB			Unknown	

Figure 1. *Chromatogram of four amino acid mixtures*

The peak of four amino acids were separated well. The retention time was 20 minutes which suitable to investigate the hydrolysis of samples on HPA-S equipment.

3.2. Survey of hydrolysis conditions with HPA-S

3.2.1 Survey of sample hydrolysis temperature program

Temperature is the most important factor determining the efficiency of hydrolysis. To select the appropriate sample hydrolysis temperature, it is necessary to survey of representative food

samples (powder milk) and animal feed (TACN - premix form), according to different temperature programs with sample weight (0.5g), the volume of HCl 6N acid (5mL), and hydrolysis time is 45 minutes. The hydrolysis programs are summarized in Table 1.

***Table 1**. Program of hydrolysis temperature of amino acid samples*

Steps	Temperature			
	Program 1	Program 2	Program 3	Program 4
Step 1	80°C - 5 min	80°C - 5 min	80°C - 5 min	80°C - 5 min
Step 2	120°C - 10 min	120°C - 10 min	120°C - 10 min	120°C - 10 min
Step 3	160°C - 10 min	160°C - 10 min	160°C - 10 min	160°C - 10 min
Step 4	200°C – 20 min	240°C - 20 min	270°C - 20 min	300°C - 20 min
Step 5	Cool to temperature of at least 40°C			

Results of the analysis of 4 essential amino acids with different hydrolysis temperature programs are presented in Figures 2 and 3.

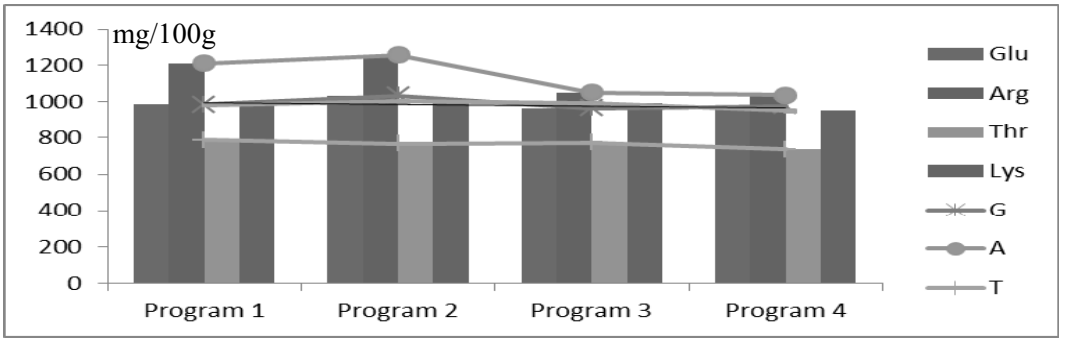


***Figure 2**. Results of hydrolysis survey of powder milk samples*

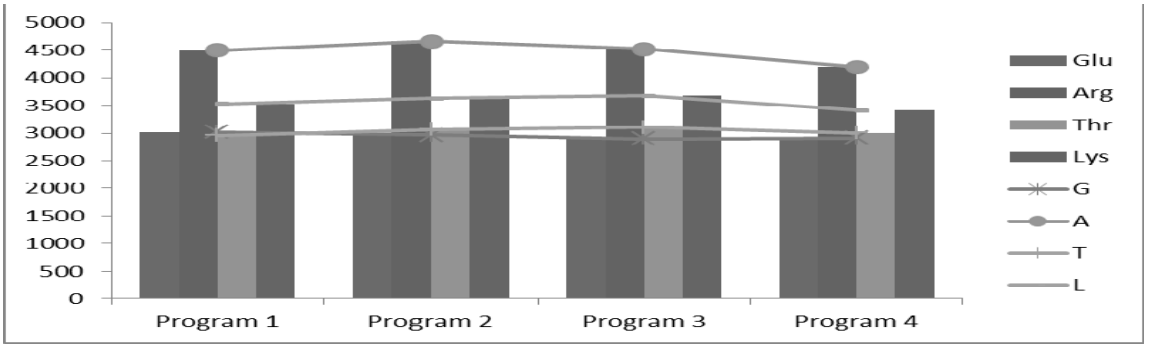


***Figure 3.** Results of hydrolysis survey of of feed samples*

The hydrolysis process underwent various heating steps to react slowly. Accordingly, in program 2 corresponding to maximum temperature of 240°C, amino acid content in the samples reached the highest and most stable values. However, there has not been so much change when increasing the temperature in the range of 200 to 300°C (the reduction of content at less 20% with Arg). Therefore, the maximum hydrolysis temperature was selected at 240°C and remains fixed for the next survey steps.

3.2.2 Survey of sample hydrolysis time

The time of sample hydrolysis was investigated after the maximum sample hydrolysis temperature of 240°C was selected. Hydrolysis needs to be changed in the last step of the

temperature program before running the cooling process within about 10 - 30 minutes. The specific conditions are shown in Table 2.

Table 2. *Survey of hydrolysis time of amino acid samples*

Steps	**Temperature**			
	Program 1	Program 2	Program 3	Program 4
Step 1	80°C - 5 min	80°C - 5 min	80°C - 5 min	80°C - 5 min
Step 2	120°C - 10 min	120°C - 10 min	120°C - 10 min	120°C - 10 min
Step 3	160°C - 10 min	160°C - 10 min	160°C - 10 min	160°C - 10 min
Step 4	200°C - 10 min **Total: 35 min**	240°C - 15 min **Total: 40 min**	240°C - 20 min **Total: 45 min**	240°C - 30 min **Total: 55 min**
Step 5	Cool to temperature of at least 40°C			

Results of analysis of 4 essential amino acids with different hydrolysis time programs are given in Figure 4 and Figure 5.

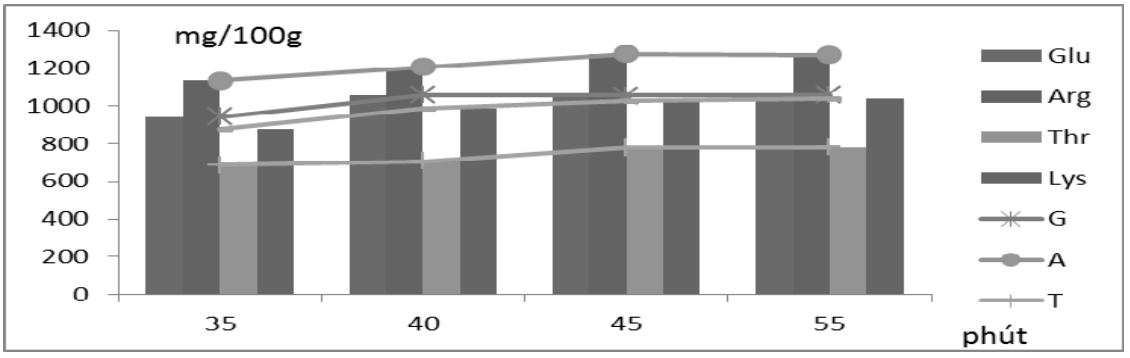


Figure 4. *Survey results of hydrolysis time of powder milk samples*

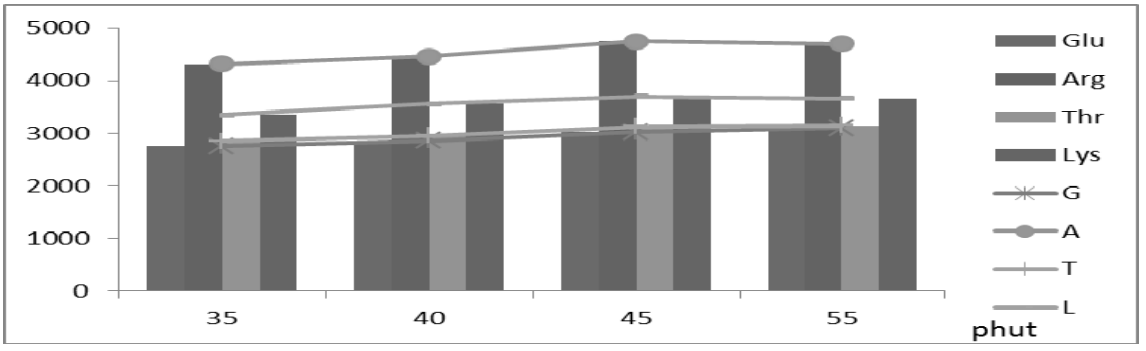


Figure 5. *Survey results of hydrolysis time of feed samples*

The results revealed that the amino acids content tended to rise when the hydrolysis time increases, and reached the maximum time at 45 minutes for hydrolysis process. After that, the amino acids content turned out to be stable, not significantly changed despite extended hydrolysis time. Therefore, the optimal sample hydrolysis time is selected as long as 45 minutes.

3.2.3 Survey of sample weight

Study on the hydrolysis response of HPA-S system was undertaken different sample weight in the range of 0.1 to 1.2 g, with the conditions of time and hydrolysis temperature selected on a powder milk sample (20% protein) and a feed sample (60% protein). The results are given in Figure 6 and Figure 7.

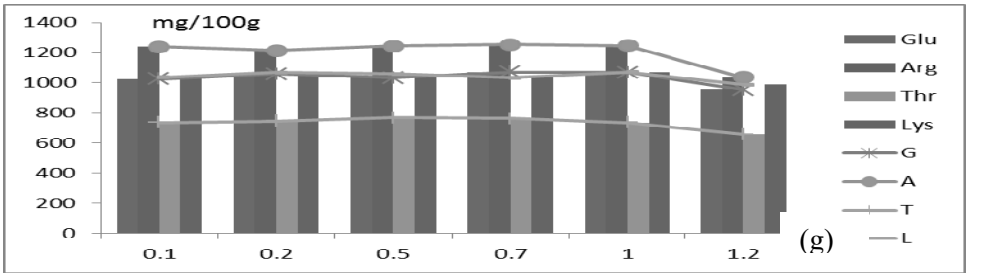


Figure 6. *Correlation of amino acids content and milk samples mass*

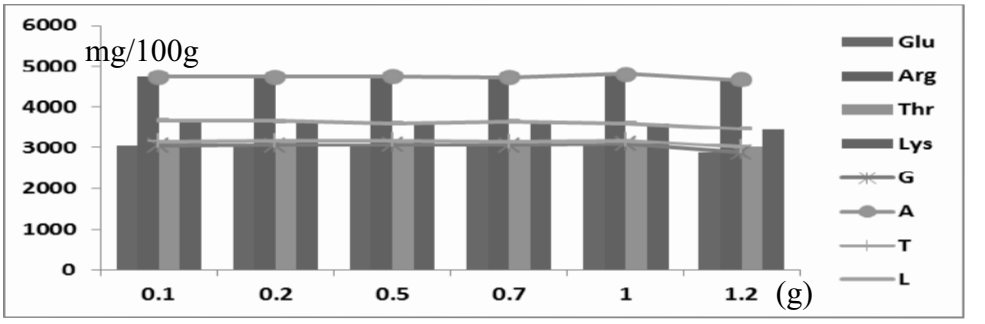


Figure 7. *Correlation of amino acids content and feed samples mass*

The survey results of the mass of hydrolyzate samples based on powder milk and feed samples with protein content of about 20 - 60% indicated that, when the weight of the sample increased gradually from 0.1 to 1g, the content of amino acids remained tend to be stable (results range within ± 5%). However, with the weight of the sample of 1.2 g, the amino acids content tended to decrease sharply (the highest decrease of 20%). Therefore, the weight content of the samples should not exceed 1g, unless there is a further study of the concentration and amount of HCl acid using sample hydrolysis.

3.3. Sample processing process

Under the survey conditions, the procedure for determining amino acids after hydrolysis on HPA-S is summarized in Figure 8:

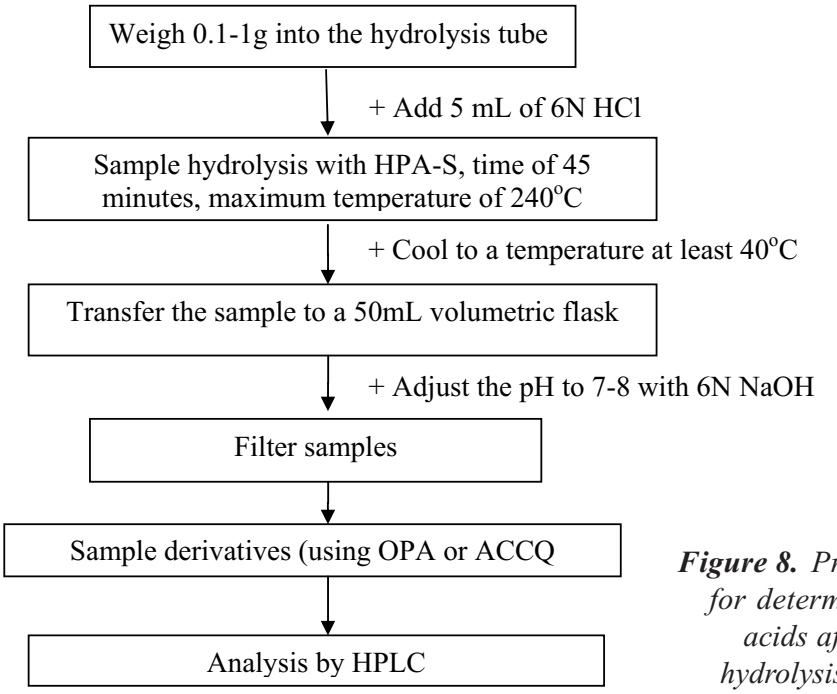


Figure 8. *Process diagram for determining amino acids after sample hydrolysis with HPA-S*

Comparision between two samples treatment process by using HPA-S and oven, due to the operating principle and structural characteristics of the HPA-S system, it is possible to increase the temperature to 240^{0}C to completely break down protein peptide bonds within a short time (about 45 minutes). In addition, the hydrolysis system is completely sealed for better repeatability and stability.

3.4. Evaluating hydrolysis process by using HPA-S and oven according to routine method at NIFC

The results of amino acid analysis based on the hydrolysis process using HPA-S and oven currently applied according to the routine method at NIFC were evaluated. Several food and feeding samples were selected, simultaneously analyzed 17 amino acids according to the pre-column derivative process with ACCQ reagent, and analyzed four essential amino acids using derivatives OPA by HPLC, then each pair of experimental data by a paired t-test could be compared. The analytical results are summarized in Table 4 and Table 5.

Table 4. *Results of evaluation of amino acid analysis samples using ACCQ derivatives*

Amino acids	***Pork meat (mg/100g)***			***Powder milk 1 (mg/100g)***			***Feeding 1 (mg/100g)***		
	HPA-S	Oven	d_i (%)	HPA-S	Oven	d_i (%)	HPA-S	Oven	d_i (%)
Glutamic acid	1,705	1,551	9.40	453	412	9.50	4,884	4,275	13.3
Arginine	1,131	1,075	5.17	816	776	5.15	2,967	2,846	4.16
Threonine	774	866	11.3	851	953	11.3	2,484	2,676	7.44
Lysine	1,738	1,999	13.9	1,582	1,820	14.0	4,320	4,779	10.0
T_{cal}	3.15			2.26			3.02		
T_{theory} *(P=0.95; f=3)*	3.18			3.18			3.18		

Table 5. *Results of evaluation of amino acid analysis samples using OPA derivatives*

Amino acids	***Powder milk 2 (mg/100g)***			***Functional food (mg/100g)***			***Feeding 2 (mg/100g)***		
	HPA-S	Oven	d_i (%)	HPA-S	Oven	d_i (%)	HPA-S	Oven	d_i (%)
Glutamic acid	705	668	5.40	1,774	1,657	6.80	4,325	4,468	3.30
Arginine	620	634	2.20	1,866	2,065	10.1	4,522	4,372	3.41
Threonine	790	695	12.8	1,363	1,323	3.01	3,765	3,689	2.10
Lysine	758	702	7.71	2,184	1,879	15.0	3,629	3,532	2.72
T_{cal}	2.94			2.91			0.69		
T_{theory} *(P=0.95; f=3)*	3.18			3.18			3.18		

The analyzed results of food and feed samples in Table 4 and Table 5 all give the value of $T_{cal} < T_{theory}$, demonstrating that two methods of hydrolysis using oven and HPA-S with different sample backgrounds give similar results. Therefore, it may be concluded that the sample hydrolysis method with HPA-S can replace the sample hydrolysis method with an oven to determine the content of amino acids, to shorten sample preparation, but still ensures stability and high accuracy.

4. CONCLUSIONS

Evaluation the results of amino acids analysis on food and feed samples by two hydrolysis

procedures (HPA-S and oven) showed quite similar results. However, the hydrolysis process using HPA-S shortens the sample preparation and provides higher stability.

The study has contributed to the proposal of a new method of treating amino acid analysis samples that can be applied at the NIFC. In addition, this study gives a chance to develop the application of sample preparation by HPA-S for analyzing other substances apart from metal elements.

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Tóm tắt

NGHIÊN CỨU QUY TRÌNH THỦY PHÂN MẪU THỰC PHẨM, THỨC ĂN CHĂN NUÔI BẰNG THIẾT BỊ TRO HÓA MẪU ÁP SUẤT CAO (HPA-S) NHẰM XÁC ĐỊNH ĐỒNG THỜI HÀM LƯỢNG MỘT SỐ ACID AMIN BẰNG HPLC

Đinh Viết Chiến, Nguyễn Tiến Luyện, Nguyễn Thị Hồng Ngọc, Phạm Công Hiếu, Đỗ Tất Thành, Phạm Thu Giang, Tô Quốc Tường, Nguyễn Thị Lan, Dương Minh Tuấn, Doãn Văn Kiên

Viện kiểm nghiệm an toàn vệ sinh thực phẩm Quốc gia

Nghiên cứu này phát triển quy trình thủy phân mẫu thực phẩm và thức ăn chăn nuôi bằng thiết bị tro hóa mẫu áp suất cao (HPA-S) nhằm xác định một số acid amin bằng sắc ký lỏng hiệu năng cao (HPLC). Thiết bị HPA-S được sử dụng chủ yếu trong xử lý mẫu cho phân tích các kim loại bằng quang phổ. Tuy nhiên, nghiên cứu cũng cho thấy HPA-S có thể được sử dụng trong việc thủy phân các mẫu thực phẩm và thức ăn chăn nuôi nhằm mục đích phân tích các acid amin. Thiết bị HPA-S có thể đạt nhiệt độ tối đa tới 300^{o}C, và duy trì liên tục ở áp suất 130 bar, giúp phá hủy hoàn toàn những nền mẫu phức tạp nhất. Việc nghiên cứu thành công ứng dụng của thiết bị HPA-S để thủy phân mẫu phân tích các acid amin làm rút ngắn đáng kể thời gian của bước chuẩn bị mẫu, mà vẫn cho độ ổn định tương đương, thậm chí cao hơn các phương pháp thủy phân mẫu thông thường.

Từ khóa: *thực phẩm, thức ăn chăn nuôi, acid amin, HPA-S, thủy phân, HPLC.*